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Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Third Meeting
March 25-27, 2002

Meeting Summary

Final

On March 25-27, 2002, the U.S. Environmental Protection Agency (EPA) convened the third meeting of the EDMVS. The meeting objectives included:

- Understand the practical aspects of the implementation of the validation process by the Endocrine Disruptor Screening Program (EDSP).
- Provide comments and advice on:
  - The suite of chemicals used in prevalidation of EDSP-related assays;
  - The fish reproduction assay detailed review paper;
  - Special studies on fathead minnow assays, vitellogenin assay, and avian dosing protocol;
  - The aromatase detailed review paper;
  - The *in utero* through lactation assay protocol.
- Understand sources of data used in assessing human health effects.
- Understand EPA's approach to addressing low dose issues.

Copies of presentation slides and other materials distributed at the meeting may be obtained by contacting Jane Smith at smith.jane-scott@epa.gov or 202/564-8476. Many of the materials also are available on the EPA website at http://www.epa.gov/scipoly/oscpendo/edmvs.htm EPA has established an administrative record for this meeting under docket control number OPPTS-42212E. The docket is available for inspection in the TSCA Nonconfidential Information Center, North East Mall Rm. B-607, Waterside Mall, 401 M St., SW., Washington, DC. The center is open from noon to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number of the center is (202) 260-7099.

Monday, March 25, 2002

I. Welcome and Opening Comments

Vanessa Vu, EDMVS chair and director of the EPA Office of Science Coordination and Policy (OCSP), welcomed the EDMVS members and the public to the meeting. She noted that significant strides had been made at the two previous meetings and thanked EDMVS members for their continuing invaluable advice.

II. Introductions, Agenda Review, and Review of Previous Meeting Summary

Paul De Morgan, senior mediator with RESOLVE, introduced himself and asked the EDMVS members to identify themselves and their organizations.

Jane Smith, designated federal official for the EDMVS, explained that the meeting was being held in accordance with the Federal Advisory Committees Act (FACA) and all materials distributed would be available through the docket. She invited anyone with comments on EDMVS membership or other concerns to contact her.

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Mr. De Morgan gave an overview of the materials distributed to the members and reviewed the meeting agenda. He noted that time was allotted for public comment at the end of the first and second days of the meeting. Mr. De Morgan then reviewed the meeting ground rules.

Regarding the December 2001 meeting summary, Mr. De Morgan reported that the version in the EDMVS meeting materials was similar to the previous version emailed to the subcommittee in January. He pointed out the four changes made based on comments received from EDMVS members. Mr. De Morgan also noted that, before closing discussion on the summary, he needed to speak with one member who submitted comments. Assuming that any suggested changes from this member are not raised on the second day of the meeting, the subcommittee approved the summary and revisions.

III. Review of EDMVS Work Plan

Ms. Smith presented the subcommittee work plan, revised since the December meeting to reflect changes in the projected study timelines and EDMVS members' preference for conveying more specific information. (As indicated above, copies of slides from Ms. Smith's presentation, "EDMVS' Draft Work Plan," may be obtained from the docket or EPA website.) She stated that EPA would distribute a timeline of relevant dates, as requested by the subcommittee, at the June 2002 meeting.

A member questioned where in the work plan the EDMVS would be asked to consider the tests as an entire battery. Ms. Smith indicated that the current goal would be to review the battery in October 2003, noting that the schedule depends on the timely completion of laboratory work.

Ms. Smith noted that the work plan did not specify who has the lead for each assay. She also clarified that specific dates were not given for the 14-day intact male alternative assay because it is a test for which industry is taking the lead. Gary Timm, OSCP, EPA, explained that EPA plans to develop papers on and compare the adult male, pubertal male, and male 407 assays.

Another member's questions dealt with the uterotrophic assay. Mr. Timm informed the group that the Organization for Economic Cooperation and Development (OECD) has agreed to conduct an independent peer review for this assay. For the entire battery, he indicated that a Science Advisory Panel would likely be used, which is a different process than a typical peer review. The member's second question dealt with the possible need for more time to add chemicals to the current list of seven being used for the uterotrophic assay. Mr. Timm expressed that EPA will be looking for sufficient data to compare across chemicals, and that gaps could be filled in with further testing. Ms. Smith conveyed that some aspects of the work plan are yet to be determined as information is gathered in laboratories and as subcommittee members share their input.

IV. Presentation and Discussion of the Practical Aspects of the Validation Process by the EDSP

Gary Timm, OSCP, EPA, presented the EPA validation approach to the EDMVS. (As indicated above, copies of

slides from Mr. Timm's presentation, "EPA's EDSP Validation Strategy," may be obtained from the docket or EPA website.) Mr. Timm identified and defined key terms and steps of the validation process, including relevancy, reliability, detailed review paper, prevalidation, validation, and peer review. He concluded by framing issues for discussion at this and future meetings and then took questions from the subcommittee.

Members addressed the issue of whether one laboratory is sufficient for prevalidation. A member expressed that the number of labs needed should be assay-specific and depend on how familiar the agency is with a particular assay. More labs may be needed for assays with which there is limited experience. Another member voiced a preference for using a large number of chemicals in a single lab, since transferring into additional labs may raise concerns due to differences in chemical preparation, vehicle supply and stock/strain of the animals, and the laboratory environment. Guidelines for these variables may be necessary in the future.

The EDMVS also commented on using three laboratories as a minimum for validation. A member approved of three as a sufficient number for most validation studies, but noted that no general rule is possible. Fewer than three laboratories may be appropriate in some cases, and certain studies may need additional sites.

An additional lab-related question related to whether laboratories should receive training from a lead lab or remain 'naive' with respect to the protocol. One member commented that test guidelines for labs are important, including key parameters and techniques. This member also shared that videotapes have been useful for training technicians in the past, while another noted that hand-training individuals is probably not practical.

The subcommittee also addressed the validation of the tier 1 screening battery. One EDMVS member emphasized the importance of developing criteria to determine when a test is prevalidated or when a protocol is validated, such as a statistical measure of performance. Another member saw the purpose of the studies as determining the variation inherent in each. This member expressed that performance criteria cannot always be determined in advance since acceptable levels of variability are not known before the data has been collected. Mr. Timm responded that it could be beneficial for EPA to develop performance criteria, though they would need to be assay-specific. EPA proposed to empirically validate assays on an individual basis rather than looking at the battery as a whole. An EDMVS member stated that the validation of the entire battery should be a goal, but may not be possible. EPA must recognize that additional studies may be necessary.

Regarding the desired role of EDMVS in reviewing special studies, a member suggested that EDMVS involvement should not slow the process, but the subcommittee needs to be presented with pertinent information. If members are given sufficient time to review documents sent by email, they could submit comments without formally addressing the issue during a meeting.

During the prevalidation discussion, members made the following additional comments:

- The same chemicals should not be used in both prevalidation and validation, and the number of chemicals should differ depending on the tier.
- Negatives and compounding chemicals should be included to attain sensitivity and specificity.
- Duplicative endpoints should be avoided.
- Closed formula diets of with soybean meal and alfalfa can have confounding effects, which could potentially mask low-dose effects or weak-acting compounds.
- In test guidelines, EPA may want to consider specific strain and stock to be used for an endpoint. If flexibility is introduced into a study, scientific rationale for this decision may be needed, along with testing to determine the effects of that flexibility.
- Tier 2 tests should be ready when the tier 1 screens are validated. The mammalian two-gen test has been requested by industry for a long time. It is a long-term protocol, and adding endpoints will lengthen the time needed. EPA responded that they are conducting one-gen studies to investigate chemicals and are planning to address this test.
- When investigating phytoestrogens in feed, the thyroid should be investigated.
- When EPA decides on a standard chow (e.g., 5002), they should explain the rationale for the choice to the subcommittee.

V. Presentation: Chemicals Used in Prevalidation of EDSP-Related Assays

Jim Kariya, OSCP, EPA, presented a table of chemicals from which to choose a core set of chemicals to be tested in all assays during the prevalidation stage. (As indicated above, copies of slides from Mr. Kariya's presentation, "Choosing Core Chemicals to Use in Pre-validation Studies," may be obtained from Ms. Smith.) He explained that the presentation was intended to set the stage for more substantive discussion of the table and its implications for EDSP on the second day of the meeting. He noted that the full core set would not be tested in special studies, and certain core chemicals would not be tested in assays for which there is no need to test them (e.g., no need to test thyroid chemicals in an aromatase assay). He also noted that the core set does not preclude testing other chemicals if necessary for a particular assay. He explained that the purpose of having a set of core chemicals is to compare the same chemicals across all assays and to establish the ability of assays to respond to different modes of action. Mr. Kariya listed several criteria for choosing the set of chemicals: coverage of estrogen, androgen, and thyroid; mode of action; potency; and applicability to both humans and ecology. He said that additional considerations include solubility, availability in pure form, stability, and whether to include "negative toxics," chemicals that cause an effect but not by an endocrine-related mechanism.

Mr. Kariya explained that the information regarding chemicals in the table "Chemicals Used in Prevalidation of EDSP-Related Studies" only covered work done by the EDSP on those chemicals, and not all studies in the literature.

Following the presentation, individual members offered suggestions for refining the table:

- Include actual values rather than classify the chemicals as "weak" or "strong."
- Footnote where there are cross-over or compounding issues.
- Include a disclaimer that the chemicals are being used for testing purposes and being on the list does not mean a chemical poses a risk to humans or is an endocrine disruptor.
- Include information from the literature.
- Include a subclassification to differentiate between the imprecise and highly precise modes of action.
- Include the Chemical Abstracts Service (CAS) numbers.

Several members suggested specific corrections for the 'comments' column. Some recommended either including references or removing the comments entirely. Others suggested that the comments column be used just to indicate what the chemical is (e.g., fungicide, pharmaceutical aid). One member suggested keeping the comments that indicate if a chemical is a known agonist.

Dr. Vu noted that the purpose of the table was to stimulate discussion and provide information for choosing a set of core chemicals. A member commented that the table could serve as a valuable summary of everyone's knowledge on the chemicals listed. Mr. Kariya reminded the EDMVS members that the discussion would continue at the end of the next day.

VI. Overview of Ecotoxicological Assays

Les Touart, OSCP, EPA, presented an overview of the ecotoxicological assays to put them in context and provide an update on their status. (As indicated above, copies of slides from Dr. Touart's presentation, "Overview of Ecotoxicological Assays," may be obtained from the docket.) He outlined the ecotoxicological assays recommended by EDSTAC and commented that the purpose of including diverse taxa in the screening battery is to reduce the likelihood that important pathways for metabolic activation or detoxification of the test substances are missed.

Dr. Touart outlined the key features and proposed validation timeline for the tier 1 ecotoxicological assays (short-term fish reproduction and amphibian metamorphosis) and the tier 2 ecotoxicological assays (fish life cycle, avian two-generation, amphibian development and reproduction, and mysid life cycle). He noted that EPA will lead the OECD activities for the fish and bird assays.

In response to a question, Dr. Touart commented that the primary purpose of tier 1 is to determine whether a substance has estrogen, androgen, or thyroid activity.

VII. Presentation and Discussion of Special Study: Tier 2 Avian Dosing Study

Dr. Touart said that the avian dosing study plan is the plan recommended by the OECD avian expert group to evaluate alternative exposure scenarios. (As indicated above, copies of slides from Dr. Touart's presentation, "Avian 2-Generation Dosing Study Plan," may be obtained from the docket.) He explained that the context of the avian two-generation assay is to assess the reproductive viability of offspring, and endocrine endpoints (gross morphology and histology, developmental landmarks, and plasma and fecal/urate hormones) have been added. He said the objectives of the study are to assess the importance in timing of treatment to the parent generation and to evaluate whether the offspring generation should receive dietary treatment. He said that the recommended test substances for the study are 17β-estradiol and methyl parathion.

Some members commented on the logistical challenges of the study and the difficulties of working with Japanese quail. One member cautioned that interaction with humans can affect quails' sexual maturation. Dr. Touart replied that the practicality of the various endpoints would be discussed in the detailed review paper. Other members commented on the complexity of the study due to the large number of animals and endpoints.

A member suggested adding a thyroid-active chemical to the study. Dr. Touart said that they had chosen not to include thyroid because of the complexity of the study even with only two substances. He said that when a preferred protocol is chosen, a multichemical analysis will be done.

Noting that the proposed dosing covers less than an order of magnitude, a member suggested that the study should be designed to detect the no observed adverse effect level (NOAEL). Another member suggested that EPA consider doing benchmark doses and going beyond NOAEL.

VIII. Public Comment

At the conclusion of the day's deliberations, members of the public attending the meeting were given the opportunity to provide comments. Mr. De Morgan indicated that each person's comments would not be captured verbatim in the meeting summary, but rather just briefly summarized. He encouraged all to submit their comments in writing to Ms. Smith for inclusion in the EPA docket and posting on the website.

Rick Becker, American Chemistry Council
Dr. Becker shared comments on the chemicals to be used in prevalidation of EDSP-related Assays. (As indicated above, copies of slides from Dr. Becker's presentation, "Comments on 'Chemicals Used in Prevalidation of EDSP-Related Assays,'" may be obtained from the docket.) Dr. Becker commented that the table presented by Mr.

Kariya should include a disclaimer that inclusion of a substance in the table does not mean that use of the chemical poses a risk or that the chemical is an endocrine disruptor. He said that each entry in the table should be referenced and noted some specific errors and omissions in the information the table presented.

Kristin Brugger, Crop Life America
Ms. Brugger presented comments on the avian dosing study. (As indicated above, copies of slides from Ms. Brugger's presentation, "USEPA Avian Dosing Study: Crop Life America Comments," may be obtained from the docket.) Ms. Brugger asked EPA to consider the need for and design of the protocol. She suggested that the approach should use relevant fitness endpoints only and should look for reproductive impact consistent with endocrine mechanism. She also offered several specific suggestions for improving the study plan.

Katie Holmes, Crop Life America
Ms. Holmes commented that the short-term fish reproduction assay does not meet all of the EDSTAC criteria for screens and asked EPA to reevaluate the assay. She also commented that data from the fathead minnow assay may be difficult to interpret.

Phil Zahodikin, CRC Press
Mr. Zahodikin noted that EPA will issue a new list of endocrine disruptors in December and requested that the agency expand and improve communication through its website prior to issuing the list.

Ellen Mahaich, Rhodia, Inc.
Dr. Mahaich reviewed some of the activities of the OECD Eco Validation Management Group (Eco-VMG). She suggested that the EDMVS should recommend greater involvement of additional avian expertise before the avian dosing study is undertaking. She said that the documents, comments, and concerns from this meeting should be raised to OECD before decisions are made or a path forward is chosen. She noted that EPA had provided the documents on the ecological assays to the OECD Eco-VMG. She expressed concern, however, that work on the assays would begin before the group could meet to discuss them.

Troy Seidle, People for the Ethical Treatment of Animals
Mr. Seidle expressed concern about the number of animals being proposed for use in the prevalidation studies. He commented that EPA's commitment to reducing animal use, refining procedures involving animals to make them less stressful, and replacing animals where scientifically appropriate must apply to all stages of EPA's work. He said that EPA's usual approach to using animal data in assessing human health effects is not acceptable. He encouraged EPA to consider *in vitro* testing methods.

Tuesday, March 26, 2002

IX. Presentation and Discussion of the Short-Term Fish Reproduction Assay and the Fish Screening Assays Detailed Review Paper

Dr. Touart summarized key information from the Fish Screening Assays Detailed Review Paper (DRP). (As indicated above, copies of slides from Dr. Touart's presentation, "Fish Screening Assay Detailed Review Paper" may be obtained from thedocket.) He said that the DRP was based on a review of 500 papers selected from among 10,000 records located through an on-line literature search. He added that the analysis also included consultation with experts and an internal peer review. He explained that the analysis focused on three species identified as the species of choice by the nations of the OECD: fathead minnows, zebrafish, and Japanese medaka. He outlined the known endocrine differences in fish that necessitate a fish reproduction screening assay:

- Fish differ in steroid profiles from mammals;
- Estrogen receptor in fish differs structurally and functionally from mammals;
- Steroid receptors in eggs and hepatic vitellogenin have no known analogous receptors in mammals.

Dr. Touart then reviewed the characteristics of fathead minnows, Japanese medaka, and zebrafish and listed the strengths and weaknesses of each as a test species. He said that the measurement endpoints for the assay include growth and morphological alterations, biochemical measures, and measures of reproductive performance. He outlined the three candidate protocols that emerged from an OECD Eco-VMG workshop: 21-day reproductive assay, 14-day fish reproductive assay, and 14-day fish non-reproductive screen. Dr. Touart closed by listing several significant data gaps.

A member commented that OECD considered the 14-day assay the recommended approach for a mechanistic screen and the fish reproduction assay was a tier 2 test because it included reproductive endpoints. Dr. Touart disagreed and stated that while members of the OECD Eco-VMG held differing opinions, the Eco-VMG collectively agreed to pursue both the 14-day assay and the fish reproduction assay as candidates for a tier 1 screen. In addition, Sweden has recently proposed a new 42-day zebrafish assay for consideration as a tier 1 screen.

Gary Ankley, Midcontinent Ecology Division, National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development (ORD), EPA, presented information on the short-term fish reproduction assay. (As indicated above, copies of slides from

Dr. Ankley's presentation, "A Short-Term Test for Detecting EDCs Using a Small Fish Model," may be obtained from the docket.) He offered two reasons for screening endocrine disruptive chemicals (EDCs) with fish: 1) widespread effects due to EDCs could be affecting this class of animals, and 2) fish possess unique receptors, steroids, and reproductive processes potentially not captured by other proposed screening assays.

Dr. Ankley outlined the proposed gonadal recrudescence assay and noted some of its drawbacks, including logistical and practical challenges and the potential that response will be protracted and highly asynchronous. He then outlined the alternative, short-term reproduction assay, noting that it includes a similar suite of endpoints to the proposed recrudescence assay. He said that the goal of the program was to develop and characterize a short-term reproduction assay with the fathead minnow for identifying (anti-) estrogens and androgens, and modulators of steroid metabolism.

After summarizing the general experimental design and noting some relevant considerations in measuring steroids, Dr. Ankley presented NHEERL's findings on the characterization of the assay with known EDCs. In general, NHEERL has demonstrated that the fish reproduction assay is responsive to estrogenand androgen agonists and antagonists and aromatase inhibitors. In closing he listed unresolved issues and research needs, including methods for dietary exposures, development of a "fingerprint" for additional EDCs and non-EDCs, and evaluation of the potential for comparable results with more abbreviated designs.

A participant asked Dr. Ankley whether the fish reproduction assay identified any environmental chemicals active through the estrogen, androgen or thyroid receptor that would not be identified by the existing mammalian assays. In response, Dr. Ankley said that in terms of environmental exposure, there would probably be EDCs that would be detected only in these non-mammalian assays. He said that in regard to modes of action, unique pathways exist but he did not know whether there were any chemicals that would hit those. Dr. Ankley indicated that the few chemicals used so far in the fish reproduction assay were active in mammalian assays and that as the assay is further explored, additional chemicals will need to be assessed. He commented that when these data are together it will befor the EDMVS to decide whether any EDCs would be missed if the battery included only mammalian assays. He added that if the short-term fish assay performed well it potentially could make some of the other proposed assays unnecessary.

A member commented that the assay is too long and expensive to be considered a screen. He said the assay had crossed from being a screen for estrogen, androgen, and thyroid (EAT) activity to being a reproductive screen. He urged EPA to return to a shorter assay. He advised that the diagnostic and apical endpoints should not be included and the assay should focus on endpoints specific to EAT activity. Another member also noted that the assay included two diverse sets of endpoints meant for different purposes. He commented that the question to answer is, given the uterotrophic and Hershberger assays, what is needed from a fish assay. A third member commented that at this stage, including diverse endpoints helps to link mode of action to toxicity. She observed that the question will be whether the assay described by Dr. Ankley will be the protocol. A member responded that correlating endpoints with adversity is beyond the scope or the standardization and validation program. He said that the goal for a screen is to be sensitive and specific, in the shortest amount of time.

A member commented that length of time should not be the definitive criteria for determining whether an assay is a screen or a test. He agreed with the other members who commented that an important consideration is whether the assay provides necessary data. He noted that Dr. Ankley had highlighted some aspects unique to non-mammals that could add important

information to the screening stage.

A member commented that the technical document (the NHEERL report) was ambiguous in regard to appropriate dosing levels for the range-finding tests. He acknowledged the difficulty of writing the section without ambiguity but suggested that EPA should provide some guidance to help get researchers "in the ballpark" for the dosing regime.

A member asked how this assay might fit with tests required for pesticides under other regulations. Dr. Ankley commented that there is not a cost-effective reproduction test for fish that is done routinely. He said that an assay like this one might complement the early lifecycle development test required under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) or might be useful for other regulations as well.

Dr. Touart asked members to send him their comments, if any, on the DRP itself.

X. Presentation and Discussion of Special Study: Comparative Evaluation of Fathead Minnow Assays

Dr. Touart explained that the objectives of the special study are to evaluate the sensitivity and transferability of short-term reproduction assay with fathead minnow and to conduct a side-by-side comparison of the 21-day reproduction assay, the 14-day reproduction assay, and the 14-day non-spawning assay. (As indicated above, copies of slides from Dr. Touart's presentation, "Comparative Evaluation of Fathead Minnow Assays" may be obtained from the docket.) He said that the study will use fadrozole, methoxychlor, methyl testosterone, and flutamide and will include growth and morphological alterations, biochemical measures, and measures of reproductive performance as measurement endpoints. Noting that the assay looks at a pattern of responses, Dr. Touart commented that generally the intent of the special study is to determine how much time is necessary to evaluate various endpoints.

Specific advice from individual members included the following:

- Verify the concentration in exposure at least daily, depending on the compound. Dr. Ankley noted that the proposed chemicals have all been tested and are fairly stable.
- Think in advance about data interpretation and how best to set up the data so that conclusions may be drawn on the comparison of the assays.
- Include a thyroid active compound.
- Develop a statistical plan for how to compare the results from the contract lab to those from the EPA lab since there will not be a one-to-one correspondence of doses.
- Consider using trenbolonerather than methyl testosterone as methyl testosterone must be mixed, which may increase variability.
- Clarify that tissue residue data should be pooled.

XI. Presentation and Discussion of Special Study: Comparative Evaluation of Vitellogenin Methods

Dr. Touart explained that the overall purpose of the study is to coordinate an interlaboratory

comparison of vitellogenin (VTG) analytical methods. He outlined specific objectives:
- Prepare a standard evaluation series of fish plasma and tissue;
- Identify laboratories to participate and coordinate transfer of samples;
- Collate and analyze results.

He said that VTG would be measured through indirect quantification of VTG protein, direct quantification of VTG protein, quantification of VTG messenger ribonucleic acid (mRNA), and mass spectrometry.

In response to questions, Dr. Touart explained that the processes in Japan and Europe to examine other species are not parallel to EPA's process on fathead minnows. He said that the OECD Eco-VMG is coordinating the work among countries. He added that depending on the results of EPA's comparative evaluation, comparisons might not be done on zebrafish and Japanese medaka. He noted that the study plan protocol has been left open to allow medaka and zebrafish to be incorporated.

Members discussed the objectives of the study and whether the study plan would achieve those objectives. Dr. Touart clarified that the primary question being examined is performance based: will the different methods produce the same results, or will a particular method need to be specified? One member commented that the plan should explain how conclusions will be drawn from the data. She said that in particular, EPA should consider how the different methods will be evaluated if only one laboratory tests a given method, or the plan should specify that each method must be tested in at least two labs. Other members agreed that further consideration should be given to how EPA will determine whether differences in results are due to differences in the methods or differences among the labs. One member suggested doing a power analysis to determine how many labs would be necessary for the study results to be statistically valid and then doing a survey of labs to determine which could participate.

Specific advice from individual members included the following:
- Better articulate objectives.
- Provide clear performance criteria.
- Ensure adequate representation of methods.
- Discuss trade-offs (pros/cons) of the methods in the final report.
- Ensure blind samples.

XII. Presentation and Discussion of the Core Chemicals Used in Prevalidation of EDSP-Related Assays

Mr. Kariya presented two proposals developed by EPA for a set of core chemicals: a "full set" proposal including sixteen chemicals and a "limited set" proposal including seven chemicals. (As indicated above, copies of slides from Mr. Kariya's presentation, "Choosing core chemicals to use in pre-validation studies" may be obtained from the docket.) He explained that EPA preferred the limited set due to logistical and cost considerations. He said that in choosing chemicals to propose for the limited set, he tried to select those planned for the pubertal assays and he tried to cover as many mechanisms as possible. He presented the limited set as follows:
- Estrogen agonist: Methoxychlor

- Androgen inhibitor: p,p'-DDE
- Thyroid inhibitor: Phenobarbital
- Aromatase: Fenarimol
- Other steroidogenesis: Ketoconazole
- CNS/pituitary: Atrazine

Mr. Kariya explained that EPA was also considering adding a "negative toxic" to the set and listed several specific chemicals being considered. He said that the chemicals are negative toxics in that they cause a decrease in body weight gain, but their toxicity is not related to endocrine disruption. He also noted that because a reduction in body weight can cause endpoints similar to those of endocrine disruption, EPA intends to conduct a limited feeding study to examine the direct effects of weight loss. He explained that, therefore, including a negative toxic in the core set may not be necessary if it was only to test body-weight effects.

Several members commented that negative toxics serve other purposes as well and should be included. One noted that the purpose of a screen is not to find positives but to weed out negatives.

Members discussed the purpose of the set of core chemicals and the adequacy of the limited set to meet that purpose. One member commented that the purpose of the core set is to begin to compare assays and evaluate the efficiency of the battery, which would be difficult to do with only one chemical for each mode of action. Other members agreed that more chemicals would be needed to make comparisons. One member particularly noted the need for more chemicals to cover thyroid activity. Another member suggested choosing two chemicals for each mode of action, going to the extreme ends of the intermediary (weak and strong) for estrogen and androgen. A member commented that there should be a set that goes through all the assays to allow for comparison, but he noted that this did not necessarily have to be done in prevalidation. Another commented that EPA should try to cover a range of potency with the core set to help determine what level of potency is necessary to detect an effect in an assay. He suggested that a limited set of chemicals could be used in prevalidation to determine whether an assay would detect an effect and then a different set could be used in validation to challenge the prevalidation findings and test the potency range.

A member suggested using chemicals from the set selected by OECD in 1997 whenever possible. Another member cautioned, however, that that set was selected before any validation efforts had begun for the uterotrophic and Hershberger assays and may not be relevant at this point.

One member pointed out that the set of core chemicals for tier 2 validation will depend somewhat on the extent to which the tier 2 tests are modified based on what is learned from tier 1 validation. Dr. Vu commented that further discussion on the tier 2 chemicals will be needed.

Other specific suggestions from individual members included:

- Instead of central nervous system (CNS)/pituitary as a category, use neuroendocrine.
- Consider including chemicals that are common in the environment.

Members agreed that choosing chemicals for each mode of action would be a good approach. EPA offered to consider specific suggestions from the members regarding chemicals and their modes of action for testing in all assays during the prevalidation stage.

Following the discussion, Mr. Kariya asked whether members were advising that EPA delay prevalidation of the pubertal assays until the core set of chemicals was chosen. Members clarified that they were not suggesting delaying the pubertal assays as they had already reviewed the chemicals to be used in those assays.

XIII. Presentation and Discussion of Sources of Data Used in Assessing Human Health Effects

Dr. Vu made a presentation to describe ongoing activities addressing human relevancy and discuss the need for further work. (As indicated above, copies of slides from Dr. Vu's presentation, "Use of Data in Assessing Human Health Effects," may be obtained from the docket.) She outlined EPA's risk assessment guidelines and the risk assessment process for human health. She commented that the current approach:

- emphasizes full characterization and integration,
- includes maximum use of all relevant information,
- expands the role of mode of action information,
- uses harmonized approaches for all toxicity, and
- includes a two-step dose response assessment.

Dr. Vu explained the major default assumptions used in assessments. She said that in the absence of adequate human data, an agent that produces an adverse effect in experimental animal studies is assumed to pose a hazard to humans, and data from the most appropriate or sensitive species should be used. She said that in the absence of mode of action (MOA) information, a linear dose response curve is assumed for carcinogenic effects and a nonlinear dose response (or threshold) is assumed for other effects.

Dr. Vu listed current activities (by EPA and other entities) in the area of carcinogenicity:

- A framework for evaluating mode of action has been developed for carcinogenic effect;
- Several activities are underway to provide additional guidance for evaluating the relevance of the animal mode of action to humans; and
- Review of available data is underway for pharmaceuticals to determine similarities and differences between animals and humans.

Noting that the EPA interim policy on endocrine disruption is still in place, Dr. Vu outlined EPA's planned activities on endocrine disruptors. She said the activities include case studies demonstrating an integrated approach for using available data to assess effects in humans and wildlife species, a framework for risk assessment of endocrine disruptive chemicals, and a review of pesticides with common modes of action. She commented that future needs include 1) continued research and evaluation of the modes of toxic action in animals and human relevancy and 2) a demonstration of the usefulness of a framework to evaluate modes of action for non-cancer toxicity and human relevancy.

A member suggested that EPA, possibly in cooperation with other agencies, should explore the possibility of obtaining some of the large amount of information held by the agribusiness and pharmaceutical industries. He acknowledged that issues of information release may be a barrier but suggested that companies may be willing to share some information, such as data on non-economically viable products. Another member noted that if a chemical has been registered, data on it are publicly available. She added that some chemicals never make it to registration, and data on them may be useful information.

Another member commented that at some level there is a limit to the relevance of a chemical-by-chemical and MOA-by-MOA approach.

In response to questions about the relative importance of toxic effects and MOA, Dr. Vu explained that EPA prefers to understand the MOA but does not ignore toxicity if the MOA cannot be understood. She added that without understanding the MOA, scientifically sound cumulative effects cannot be determined. A member commented that the approach may vary among EPA programs.

XIV. Presentation and Discussion of EPA's Approach to Addressing Low Dose Issues

Dr. Vu read the EPA Statement Regarding Endocrine Disruptor Low-Dose Hypothesis to the subcommittee. This statement explained the findings of the National Toxicology Program (NTP) scientific peer review, that credible studies both supported and did not support a low-dose effect. Due to these conflicting findings, EPA believes additional research is needed. As such, ORD is conducting intramural and extramural research, will solicit research proposals on the issue, and will consider the EPA/NTP workshop recommendations. EPA will also monitor ongoing research to better understand low dose mechanisms and modes of action. EPA recognizes that future information may support testing on a case-by-case basis for chemicals, though believes it is premature to require that substances be routinely tested for low-dose effects.

Dr. Elaine Francis, Endocrine Disruptor Research Program, ORD, EPA, discussed likely next steps for research, including convening a panel of scientists to issue requests for applications (RfAs) from ORD, OPPTS, and other interested offices. The panel would also decide the structure of the research, including whether it would take place at a single center or several locations. Ultimately, the data would be integrated with EPA research, and a state of the science report would be issued.

Members had the following comments regarding the low-dose issue:

- Non-mammalian data has not been looked at yet and could broaden the investigation.
- EPA should be explicit aboutits bias toward type-II errors.
- It is important to reduce as many variables as possible while testing, such as using the same materials and animals. This could be achieved within the context of the RfAcriteria.
- Low-dose exposure through food could be a critical issue.

XV. Public Comment

At the conclusion of the day's deliberations, members of the public attending the meeting were given the opportunity to provide comments. Mr. De Morgan indicated that each person's comments would not be captured verbatim in the meeting summary, but rather just briefly summarized. He encouraged all to submit their comments in writing to Ms. Smith for inclusion in the EPA docket and posting on the website.

Junshi Miyamoto, Ministry of Economy, Trade, and Industry Subcommittee on Endocrine Disruptors
Dr. Miyamoto shared information on the November 17-21, 2002 International Symposium on Endocrine Active Substances in Yokohama, Japan, and invited participants to attend.

Angelina Duggan, CropLife America (CLA)
Dr. Duggan commented that CLA does not support the *in utero* through lactation assay development. She noted that CLA believes this study has a complex design, is resource intensive, does not demonstrate comparative sensitivity, and may obtain confounding results. Dr. Duggan also suggested that this assay is redundant with tier 2 and other regulatory tests, and that the same endpoints could be studied by expanding other tests. CLA recommends that EPA should focus on current EDSTAC mammalian and wildlife assays and tests.

Rick Becker, American Chemistry Council
Dr. Becker discussed the aromatase assays, commenting on the importance of decision criteria for assays. He asked EPA to reflect on the difference between categories of moderate, weak, and no activity, and the impact that each result could have in terms of triggering additional work. He recommended that EPA consider *in vivo* confirmatory studies and that the 15-day intact male assay is a good *in vivo* tier 1 screen.

Troy Seidle, People for the Ethical Treatment of Animals
Mr. Seidle commented that the *in utero* through lactation assay is animal intensive and also voiced concern about the possible redundancy of the *in vivo* tests. He urged EPA to practice the reducing/refining/replacing principles in prevalidation, validation, and implementation phases. He highlighted the importance of remaining informed about current developments and new possible methods. Mr. Seidle also conveyed his satisfaction with EPA's current policy with respect to low-dose issues.

Terry Burns-Heffner, Harlan Sprague Dawley
Mr. Burns-Heffner urged EPA to continue investigation of phytoestrogen issues. Based on customer feedback, Harlan Sprague Dawley believes animals are affected before they arrive at testing laboratories by the soy-based diet given them by breeders. He suggested that the phytoestrogenic intake of these animals may exceed human consumption by 50-100 times. Study protocol should address such research variables by considering the use of standardized diets offered by companies.

Wednesday, March 27, 2002

XVI. **Presentation and Discussion of Aromatase Detailed Review Paper**

Susan Laws, Reproductive Toxicology Division, NHEERL, ORD, EPA, outlined details of the aromatase DRP, aromatase activity, and relevant assays and their place in the tier 1 screening battery. (As indicated above, copies of slides from Dr. Laws' presentation, "Aromatase: Detailed Review Paper," may be obtained from the docket.) She explained that the enzyme complex is bound in the endoplasmic reticulum and catalyzes the conversion of androgens to estrogens. It is present in the ovary, placenta, testis, brain, bone, vasculature and adipose tissue of all vertebrates. It can have a positive effect, but can act negatively with estrogen-dependent cancers.

In the proposed tier 1 screening battery, there is no single assay to test for all effects. Thus, aromatase is currently a testing component of *in vitro*, *in vivo*, and alternative assays. Dr. Laws explained the aromatase sources and methods for measuring activity for the *in vitro* assays. One candidate assay is the human placental microsomal assay, an *in vitro* subcellular enzyme preparation. Advantages of this assay include: it is commonly used to measure aromatase inhibition; it is inexpensive and rapid; placental tissue has elevated aromatase activity; historical data is available; and recombinant human aromatase could possibly be used.

A second candidate assay is aromatase activity in cell lines (e.g., H295R cells). Some cell lines such as JEG cells express high levels of aromatase and would be useful in detecting aromatase inhibitors. Others expressing low levels of aromatase would be useful in detecting effects on aromatase induction. Advantages of this assay include: cell lines are available from theAmerican Tissue Culture Collection (ATCC); the assay has excellent sensitivity using tritiated water release; the ability of test chemicals to induce or inhibit activity can be measured; and standard cell culture techniques can be used. Disadvantages or limitations of the aromatase activity in cell lines assay include: CYP19 induction is regulated by different promoters in different cell lines so that using only one cell assay will not give universally applicable data on effects on induction; the test chemical may give a false positive if it is cytoxic; other cellular enzymes may metabolize the substrate or test chemical; there are potential solubility and cell membrane permeation problems; it would involve approximately twice the cost and time of the microsomal assay; and there are restrictions on the commercial use of cell lines from ATCC.

Dr. Laws concluded that these candidate assays both specifically measure the ability of test chemicals to inhibit aromatase activity. Tissue-specific promoters for CYP19 complicate the use of cell-based systems, however. The human placental microsomal aromatase assay was thus recommended based on its aforementioned advantages.

Finally, Dr. Laws presented the subcommittee with the following two questions:

- Given the recent reports of induction of aromatase activity and CYP19 mRNA by several environmental chemicals, is the placental microsomal assay too limited?
- Should consideration be given for the use of two cell-based systems (e.g., one with low and the other with higher constitutive expression of aromatase)?

Mr. Timm asked the subcommittee for comments on two levels: recommendations of the DRP to set the stage for prevalidation work on aromatase, and recommendations on the document itself and its adequacy in framing issues. He highlighted EPA's new technology program and the

agency's goal to include new technology in the next generation of screens and assays developed for future use.

A member asked whether there are any compounds detectable in aromatase and not the pubertal female assay. Dr. Laws responded that she was not aware of any, but that the *in vitro* aromatase test would be used if the female pubertal were removed from the battery. She explained her view that the *in vitro* aromatase assay was never intended to be used alone, but as part of the tier 1 screening battery.

Another question related to safety, availability, and variability issues involved in using human tissue for a study, as well as the feasibility of recombinant systems. Dr. Bruggemeier, Ohio State University, the author of the DRP commented that there can be difficulties in obtaining tissues, but reliability and reproducibility when using placental tissue from non-smokers has been good. Dr. Laws noted that aromatase activity can be over-expressed with certain recombinant systems, but at a level comparable to that of a cell line. Dr. Bruggemeier added that new technologies for recombinant assay systems need to be developed.

It was pointed out that each lab would need to verify the formation of tritiated water as an accurate indicator of activity.

In response to a question regarding QSAR, Dr. Bruggemeier noted that there are several labs involved in developing this technology based on known inhibitors. The protein-based work is less developed and is based on inhibitors that have been published, so all active sites may not be known.

Dr. Laws answered a question regarding the categorization of substances into weak, moderate, and strong, explaining that the distinction was based on experience with ER binding. She commented that the categories may be premature until the protocol is chosen.

Another member's question related to the sensitivity of a single cell line and whether it was sufficient to extrapolate results to a whole organism effect or whether high and low basal levels are needed. Dr. Laws specified that data is being examined for one chemical in both a high and low level cell line. This issue will continue to be examined.

EDMVS members suggested the following to EPA regarding the aromatase study:

- If using the cell-based assay, a mechanism for assessing cell cytoxicity should be added.
- Cell lines should be considered in addition to microsomal assays.
- Be cautious when expressing different promoters in different cell lines.
- Patenting could inhibit laboratories' ability to use cell lines or recombinant technology if this becomes part of the protocol. The commercial viability of a protocol and its possibility to be accepted into an OECD guideline must be considered.
- Tritiated water and substrate could be used in the same culture system, in the same incubation.
- In the tritiated assay, the cell preparations create conditions optimized for enzyme activity, which could also promote other cell activity. Careful product isolation or analysis is

encouraged until there is confirmation that microsomal and cell-based assays give comparable results.
- Induction and inhibition of production should be investigated.
- The cell line is advantageous because it can pick up additional mechanisms of action, while microsomal assays have a narrow focus.
- A set of minimum performance standards for a base of chemicals should be developed, indicating certain chemicals that are able to detect minimum levels of activity.
- Appropriate chemicals should be included in whole animal studies so they will not have to be later studied in an *in vitro* method.

Mr. Timm requested the subcommittee clarify their advice on whether EPA should go forward with the microsomal assay in prevalidation, and/or also develop recombinant assays and explore a cell-based assay system. He also noted the importance of delineating the difference between research and assay development; the development of a cell-based system cannot become an open-ended research program.

A member said that, to answer Mr. Timm's questions, the whole tier 1 screening battery must be considered. Dr. Vu expressed that the June 2002 meeting could allow for an opportunity to take a broader view than the assay-specific approach that has been necessary to date.

With regards to inhibition and induction, an EDMVS member commented that it would be preferable to have an assay that can be developed for both modes of action.

Another member suggested moving forward immediately with the validation of the aromatase assay, bearing in mind that further work is needed on cell lines. *In vivo* work should also continue.

XVII. **Presentation and Discussion of *In Utero* through Lactation Protocol**

Mr. Timm introduced the *in utero* protocol, recalling EDSTAC's interest in a screening assay to look at the *in utero* or *in ovo* stage. He mentioned that it could substitute for a number of screens, become a 1.5 generation test, or combine with a developmental toxicity screen to fit into a regulatory scheme. He voiced a desire to investigate options in order to reduce economic and animal impact when testing is required.

Dr. L. Earl Gray, Jr., NHEERL, ORD, EPA, discussed the *in utero* through lactation protocol. (As indicated above, copies of slides from Dr. Gray's presentation, "Discussion of the *in utero* lactational protocol" may be obtained from the docket.) He outlined the objectives of screening and testing for estrogen, androgen, and thyroid (EAT), as well as those tier 1 screens in the EDSTAC-recommended battery that detect EAT. Dr. Gray explained that there is concern that this recommended battery lacks an assay exposing animals during perinatal life, a stage at which the fetus is highly sensitive to endocrine disruption. As such, the *in utero* lactational assay is being explored as an alternative assay recommended by EDSTAC. He listed pros and cons of including a developmental assay in the battery and went on to describe the objectives and possible uses for an *in utero* assay, noting that it could replace other protocols in the tier 1

battery, serve as a follow-up for certain chemicals previously run in a full multigeneration test, augment the current developmental toxicity test, or be included in tier 2 testing.

Dr. Gray next summarized the proposed RTI Protocol 839 for the methoxychlor study, highlighting gavage as an acceptable route of administration and making recommendations as to how the proposed protocol could be modified, including the number of litters per group and statistical comments on body weight adjustments. He reviewed the proposed alternative tier 1 screening *in utero*-lactational protocol, the general transgenerational protocol, and the general teratology/developmental toxicology protocol, describing the endpoints for the maternal cohort, neonates, and the uterotrophic cohort. He then explained the pubertal phase, detailing further steps with the pubertal female and pubertal male cohorts. Dr. Gray also shared the expected effects of the protocol using methoxychlor at different doses. He noted other strong and weak chemicals that could be included in future studies to challenge the assay, and outlined the expected low- to mid-dose effects of potent estrogens, aromatase inhibitors, and other inhibitors of steroid hormone synthesis.

In comparing dose response curves for testosterone propionate (TP) subcutaneous in the Hershberger assay and an *in utero* study, Dr. Gray explained that the *in utero* study showed specificity but not sensitivity. For assessing diethylhexylphthalate (DEHP), however, he noted that at least three other tests require more animals and have not been as successful as an *in utero* study. He explained current histopathology guidelines and options to expand observations to known target organs and more animals, which would be important for the F1 or F2 generation. Dr. Gray also listed examples of endocrine disrupting chemicals producing reproductive malformations that are not detected in standard teratology studies or in published multigenerational studies. In conclusion, Dr. Gray asked the subcommittee to consider the appropriate place for the *in utero* lactational study in the battery.

Following the presentation, the EDMVS raised questions and discussed the presentation.

One member asked about the protocol's purpose and whether it belongs in tier 1 or tier 2. Dr. Gray responded that primary purpose is to develop the *in utero* study as a screen, based on EDSTAC's recommendation. Another objective is to develop it as an alternative test for possible longer term use.

A member questioned whether *in utero*-lactational study can be classified as a screen due to timing and cost. Another member also commented that the study design is too complex to qualify *in utero* through lactation as a screen. Dr. Gray again noted that the assay had been recommended by EDSTAC due to concerns about the lack of sensitivity for developing organisms. It will not necessarily replace other assays, but should be compared with others.

Another member clarified EDSTAC's role in this assay, explaining that it was dealt with in the Screening and Testing work group, and was suggested as a way to explore avenues of the *in utero* paradigm. This member felt this study was responsive to that suggestion, adding that it is worth pursuing as a way to cover other chemicals in the battery. Another member agreed that this study is responsive to EDSTAC's concern and emphasized the importance of including an

assay addressing developmental issues.

An EDMVS member pointed out that *in ovo* has not yet been addressed in any study.

Mr. De Morgan summarized the discussion, highlighting the following points:

- The *in utero* study was a request out of EDSTAC that is now focused on a single chemical. Currently, it is a feasibility study rather than a tier 1 screen or tier 2 test.
- EPA must make decisions and explain their rationale regarding performance criteria, funding, timing, and other screening issues as they proceed.

XVIII. Next Steps and Agenda for Third Meeting

Before the meeting adjourned on March 27, EPA staff presented a list summarizing the key points and potential action items they had drawn from the subcommittee's discussions. See attachment X.

Future Meeting Dates

- The fourth EDMVS meeting will be June 10-12, 2002.
- RESOLVE will email schedule-availability forms to members to determine the best dates for the fifth meeting.

June Agenda Items

Ms. Smith presented a list of items tentatively scheduled to be on the agenda for the June 10-12 meeting.

XIX. Closing Remarks

Dr. Vu thanked the EDMVS members and the public for attending and thanked the speakers for their presentations.